Journal of Physiology (1989), **408**, *pp.* 535–547
With 5 text-figures
Printed in Great Britain

EFFECTS OF GUANOSINE NUCLEOTIDES ON SKINNED SMOOTH MUSCLE TISSUE OF THE RABBIT MESENTERIC ARTERY

BY TAKASHI FUJIWARA, TAKEO ITOH, YASUTAKA KUBOTA* AND HIROSI KURIYAMA

*From the Department of Pharmacology, Faculty of Medicine, and *The Second Department of Oral Surgery, Faculty of Dentistry, Kyushu University, Fukuoka 812, Japan*

(*Received 3 May 1988*)

SUMMARY

1. Effects of guanosine triphosphate (GTP) and guanosine 5′-*o*-(3-thio)triphosphate (GTPγS) on mechanical properties of skinned smooth muscle tissues of the rabbit mesenteric artery were investigated.

2. In skinned muscle tissues prepared by saponin, GTP (above 100 μM) and GTPγS (above 1 μM) enhanced the Ca^{2+}-induced contraction (0·3 μM-Ca^{2+} buffered with 2 mM-EGTA) in the presence of 1 μM-ionomycin, a depletor of stored Ca^{2+}. The concentration–response (pCa–tension) relationship observed in the presence of 10 μM-GTPγS shifted to the left with no change in the maximum response evoked by 10 μM-Ca^{2+}. The action of GTP was reversible but that of GTPγS was not.

3. The enhancement of the Ca^{2+}-induced contraction by GTPγS occurred with increases in the phosphorylation of myosin light chain and in the shortening velocity as measured with the slack test.

4. GTPγS had no effect on the Ca^{2+}-independent contraction of skinned muscle tissues evoked by MgATP in Ca^{2+}-free solution (4 mM-EGTA), following treatment with rigor solution containing adenosine 5′-*o*-(3-thio)triphosphate (ATPγS).

5. The present results indicate that GTP and GTPγS enhance the Ca^{2+}-induced contraction in skinned muscle tissues due to increase in the Ca^{2+} sensitivity of contractile proteins. These enhancing actions of guanosine nucleotides on contractile proteins are discussed in comparison to those of protein kinase C.

INTRODUCTION

Ca^{2+} accumulation and release from intracellular organelles are important for Ca^{2+}-signalling events within cells, in particular the endoplasmic reticulum, including sarcoplasmic reticulum (SR) (Berridge & Irvine, 1984; Nishizuka, 1984; Rasmussen & Barrett, 1984; Abdel-Latif, 1986). The intracellular second messenger, inositol 1,4,5-trisphosphate ($InsP_3$), is now recognized as a mediator for Ca^{2+} release from the SR in smooth muscle cells of many tissues and synthesis of $InsP_3$ requires the presence of GTP for activation of the GTP-binding protein (Streb, Irvine, Berridge & Schultz, 1983; Suematsu, Hirata, Hashimoto & Kuriyama, 1984; Somlyo, Bond,

Somlyo & Scarpa, 1985; Hashimoto, Hirata, Itoh, Kanmura & Kuriyama, 1986). However, the detailed mechanism of the $InsP_3$-induced Ca^{2+} release still remains obscure.

Dawson (1985) reported that the rat liver microsomes are practically insensitive to $InsP_3$ with respect to the release of Ca^{2+}, but become responsive in the presence of guanosine triphosphate (GTP). The effects of GTP seem to require the hydrolysis of GTP and possibly the transfer of the γ-phosphate group onto the protein (Dawson, Comerford & Fulton, 1986). Using skinned smooth muscle tissues of the rabbit mesenteric artery, Saida & van Breemen (1987) supported the above view, i.e. the Ca^{2+} release from the SR induced by $InsP_3$ requires the presence of GTP. In contrast, it was also reported that GTP by itself without $InsP_3$ can induce a marked release of Ca^{2+} in the microsomal fraction or permeabilized neuroblastoma cells, and the releasing effects of $InsP_3$ and GTP are additive (Ueda, Chueh, Noel & Gill, 1986; Gill, Ueda, Chueh & Noel, 1986; Chueh, Mullaney, Ghosh, Zachary & Gill, 1987). Such observations were confirmed in the rat liver, guinea-pig parotid gland and macrophages (Henne & Söling, 1986; Hamachi, Hirata, Kimura, Ikebe, Ishimatsu, Yamaguchi & Koga, 1987). On the other hand, Hamachi *et al.* (1987) noted that Ca^{2+} release from the SR prepared from rabbit hind-leg skeletal muscle is not activated by GTP. It was also reported that GTP enhances the accumulation of Ca^{2+} into the store in cultured neuroblastoma and smooth muscle cells in the presence of oxalate (Mullaney, Chueh, Ghosh & Gill, 1987).

GTP, therefore, seems to play an essential role for synthesis of the second messenger and also to contribute to regulation of Ca^{2+} release from the SR in smooth muscle cells. However, understanding of the role of GTP on the contractile protein is limited.

The present study was undertaken to examine the effect of GTP on the contraction evoked by Ca^{2+} in skinned muscle tissues of the rabbit mesenteric artery. For this, the effects of GTP and guanosine 5′-*o*-(3-thio)triphosphate (GTPγS) were also investigated on the Ca^{2+}-induced contraction following depletion of stored Ca^{2+} following treatment with ionomycin. Furthermore, to investigate the effects of GTP on the contractile protein in detail, phosphorylation of myosin light chain (20 kD proteins of MLC; MLC_{20}) and shortening velocity (V_{max}) were also measured in the skinned muscle tissues.

METHODS

Materials

Male albino rabbits (1·8–2·2 kg) were given sodium pentobarbitone (40 mg/kg I.V.), exsanguinated and the mesenterium of the ileum region was removed. For the tension recordings, the mesenteric artery was carefully excised and opened longitudinally, under a binocular microscope, after connective tissue was removed in a dissecting chamber filled with Krebs solution. A circular strip (0·3 mm in length, 0·05–0·075 mm in width and 0·025–0·03 mm in thickness) was prepared from the bundles cut transversely with small knives. The length, width, thickness and cross-sectional area of the preparation were measured with an inverted microscope at $\times 250$ magnification using a calibrated scale. The transverse cross-sectional area was calculated assuming a rectangular cross-section. To measure the extent of phosphorylation of myosin light chain (MLC_{20}), longitudinally cut strips 12–15 mm in length, 2–3 mm in width and 0·05–0·08 mm in thickness were used. Thus, in the two muscle preparations, the surface dimension differed but the thickness was the same.

Force measurement and recording

Mechanical responses were measured by attaching a circular strip to a strain gauge (UL-2, Shinko Co, Tokyo). Response frequency of the gauge was 60 Hz. The transducer was connected to a carrier amplifier (AP-620G, NihonKohden Co., Tokyo) and the output signal was displayed on a pen recorder (Matsushita Comm. Ind., Osaka). Drift was less than 3 μN/h. The tissues were superfused in a chamber with a capacity of 0·9 ml filled with Krebs solution. The perfusate was changed rapidly from one end, while the solution already present was simultaneously aspirated off with a water pump from the other end. The resting tension was adjusted to obtain maximum contraction in 128 mM-K^+ and was not greater than 15 μN. After the maximum contraction had been recorded by treatment with 128 mM-K^+, the tissue was skinned by treatment with saponin (25 μg/ml) in a relaxing solution for 20 min. The maximum amplitude of contraction of skinned muscle tissue evoked by 10 μM-Ca^{2+} was consistently larger than that of intact muscle tissue evoked by 128 mM-K^+ (Itoh, Kuriyama & Suzuki, 1983). The contractions induced by repetitive application of Ca^{2+} deteriorated considerably and the Ca^{2+} sensitivity of the contractile proteins was lowered, but addition of 0·1 μM-calmodulin prevented the deterioration and preserved Ca^{2+} sensitivity so that the Ca^{2+}-induced contraction after several trials was similar to that evoked by the first application of Ca^{2+} (Itoh, Kanmura & Kuriyama, 1986*a*). Thus, 0·1 μM-calmodulin was applied throughout the experiments.

Measurements of the shortening velocity (V_{max})

The shortening velocity of the skinned muscle strip was determined using the slack-test procedure (Edman, 1979; Arner & Hellstrand, 1985). A contraction was initiated by application of any desired concentration of Ca^{2+}. After the tension had reached a plateau level, the strip was slackened to measure the time from length decrease to force re-development (T). When the strip was then slowly re-stretched to the initial length, L_0, 2 min was required to reach a tension level identical to that which had developed before the slackening procedure. This slack test was imposed every 4 min on a single contraction to determine the shortening velocity under unloaded conditions. The relationship between T and different length steps (L) was linear in any tested concentration of Ca^{2+} (0·3–10 μM). Therefore, V_{max} was calculated from the slope of the relationship between L and T and the immediate elastic recoil was estimated from the Y-intercept of the line which was fitted to the data using the least-squares method. Data from experiments in which the coefficient of correlation between L and T was less than 0·9 were excluded from analysis.

Measurement of phosphorylation of 20 kD proteins of myosin light chain (MLC_{20})

Muscle strips were suspended in relaxing solution containing 3 μM-A23187 for 10 min to deplete the stored Ca^{2+}. Then tissues were skinned in relaxing solution containing 25 μg/ml saponin for 20 min and washed again with relaxing solution. The skinned muscle strips were then suspended in solution containing 0·3 μM-Ca^{2+} buffered with 4 mM-EGTA for 5 min and further incubated in the presence or absence of 10 μM-GTPγS for 15 min. The strips were rapidly frozen in acetone–dry ice and allowed to reach room temperature in acetone. The strips were then homogenized in lysis buffer solution with the following composition: 1% sodium dodecyl sulphate (SDS), 10% glycerol, and 20 mM-dithiothreitol (DTT), adjusted to pH 7 with Tris. The volume of lysis buffer solution was 0·1 ml/mg dry tissue weight. Two-dimensional gel electrophoresis in the second dimension, as developed by O'Farrell (1975), was used for the resolution of MLC phosphorylation. IEF gels with 4% polyacrylamide (2·5 mm in diameter and 110 mm in length) contained 8·5 M-urea, 2% Nonidet P-40, and 2% Pharmacia carrier ampholytes (1·6% for pH 4–6·5 and 0·4% for pH 3·5–10). The homogenates (50 μl) were applied and focused at a constant voltage of 100 V for 1 h, 200 V for 2 h, 400 V for 12 h and 800 V for 1 h. After focusing, the gels were loaded onto the SDS electrophoresis unit. The SDS electrophoresis gels (140 mm in width and 2 mm in thickness) were composed of stacking gels (50 mm in height with 4% polyacrylamide in 0·1% SDS and 0·125 M-Tris-HCl at pH 6·8) and separating gels (100 mm in height with 13% polyacrylamide in 0·1% SDS and 0·375 M-Tris-HCl at pH 8·8). The gels were run at a constant current density of 20 mA in the stacking gels and 40 mA in the separating gels. The gels were stained overnight with 0·03% Coomassie Brukkuabt Blue R-250, 50% methanol and 12% trichloracetic acid, and then de-stained with 10% methanol, 7% acetic acid and 0·85% phosphoric acid. The distribution of the stained protein at 20 kD MLC (MLC_{20}) exhibited the first, second,

third and fourth spots from the higher to lower pI values. The intensities of the four spots were measured with a chromatography densitometer equipped with an automatic intergrator (CS-910, Shimadzu, Kyoto). The first area at around pI 5·5 and the second area at around pI 5·45 were measured to obtain the relative value of the MLC_{20} phosphorylation (a percentage of the second spot area/the sum of the first and second spot areas; Driska, Aksoy & Murphy, 1981; Satoh, Kubota, Itoh & Kuriyama, 1987).

Solutions

The Krebs solution used contained (mM): Na^+, 137; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·6; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134; glucose, 11·5. High-K^+ solution was prepared by replacing NaCl with KCl isosmotically. The following relaxing solution was used (mM): potassium methanesulphonate (KMs), 110; $Mg(Ms)_2$, 5·1; Na_2ATP, 5·2; ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA; Dojin, Kumamoto), 4; piperazine-*N,N'*-bis-(2-ethanesulphonic acid)-(PIPES; Dojin), 20; the pH was adjusted to 6·8 with KOH at 25 °C. Solutions of desired Ca^{2+} concentration were prepared by adding appropriate amounts of $Ca(Ms)_2$ to the relaxing solution. Ionic strength was adjusted to 0·17 M in all solutions by adding or decreasing the concentration of KMs. To prevent deterioration of the Ca^{2+}-induced contraction, 0·1 μM-calmodulin was present throughout the experiments (Paul, Doerman, Zeugner & Rüegg, 1983; Itoh *et al.* 1986*a*).

Free-ion concentrations of solutions were obtained by making use of multi-equilibrium equations and the association constants (Schwarzenbach, Senn & Anderegg, 1957; Botts, Chashin & Young, 1965; Harafuji & Ogawa, 1980; Itoh *et al.* 1986*a*), i.e.

$$[H_4EGTA]/[H^+][H_3EGTA^-] = 1{\cdot}0 \times 10^2,$$

$$[H_3EGTA^-]/[H^+][H_2EGTA^{2-}] = 4{\cdot}79 \times 10^2,$$

$$[H_2EGTA^{2-}]/[H^+][HEGTA^{3-}] = 7{\cdot}08 \times 10^3,$$

$$[HEGTA^{3-}]/[H^+][EGTA^{4-}] = 2{\cdot}88 \times 10^9,$$

$$[HATP^{3-}]/[H^+][ATP^{4-}] = 5{\cdot}56 \times 10^6,$$

$$[KATP^{3-}]/[K^+][ATP^{4-}] = 8{\cdot}0,$$

$$[K_2ATP^{2-}]/[K^+][KATP^{3-}] = 0{\cdot}6,$$

$$[KHATP^{2-}]/[K^+][HATP^{3-}] = 0{\cdot}6,$$

$$[NaATP^{3-}]/[Na^+][ATP^{4-}] = 8{\cdot}8,$$

$$[Na_2ATP^{2-}]/[Na^+][NaATP^{3-}] = 8{\cdot}5,$$

$$[NaHATP^{2-}]/[Na^+][HATP^{3-}] = 5{\cdot}0,$$

$$[MgATP^{2-}]/[Mg^{2+}][ATP^{4-}] = 1{\cdot}0 \times 10^4,$$

$$[CaATP^{2-}]/[Ca^{2+}][ATP^{4-}] = 4{\cdot}0 \times 10^3,$$

$$[CaEGTA^{2-}]/[Ca^{2+}][EGTA^{4-}] = 4{\cdot}3 \times 10^{10}.$$

Numerical solutions of a set of multi-equilibria were obtained by a computer (NEC PC8801, Nihon Electric, Tokyo).

ATPγS-containing solution was composed of (mM): Tris maleate, 20; $Mg(Ms)_2$, 2·6, ATPγS, 2; EGTA, 4; and ionic strength was adjusted to 0·17 M by addition of KMs. Associated constants of MgATPγS were assumed to be the same as those of MgATP. GTP (up to 100 μM) and GTPγS (up to 10 μM) were added to each solution without consideration of the association constants.

Drugs

Chemicals used were saponin (ICN Pharmac., Cleveland, OH, USA), ATPγS, GTP, GTPγS (Boehringer Mannheim, Yamanouchi, Tokyo), A23187, ionomycin (Calbiochem-Behring, La Jolla, CA, USA), caffeine (Wako Pharmac., Tokyo), calmodulin and $InsP_3$ (kindly provided by Dr Masato Hirata, Biochem., Dentistry, Kyushu University). The water used in this study was glass-double-distilled and all other chemicals were of the highest reagent grade.

Statistics

The measured values were expressed as the mean ± standard deviation (S.D.) and the number of observations (n). The statistical significance was assessed using Student's t test for paired or unpaired values. P values less than 0·05 were considered significant.

RESULTS

Effects of GTP or GTPγS on the Ca^{2+}-induced contraction

In skinned smooth muscle tissues, application of 0·3 μM-Ca^{2+} produced contraction, and increased concentrations of Ca^{2+} enhanced the amplitude, in a concentration-

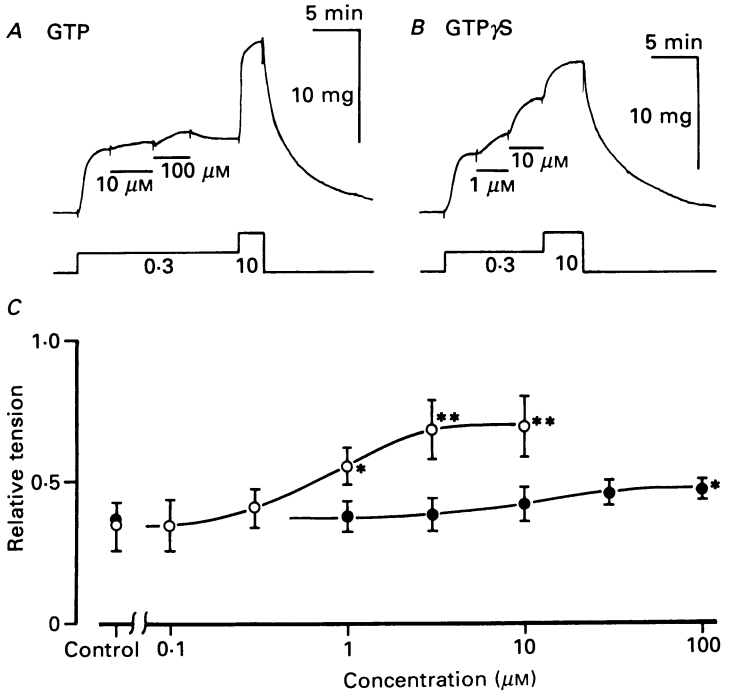

Fig. 1. Effects of GTP and GTPγS on the contraction evoked by 0·3 μM-Ca^{2+} on skinned muscle tissues of the rabbit mesenteric artery. Throughout the experiments, 1 μM-ionomycin was present. *A*, after skinning the tissues, 0·3 μM-Ca^{2+} was applied. After the tension had reached a steady level, 10 μM- and 100 μM-GTP were successively applied. As a control, 10 μM-Ca^{2+} was also applied. Baseline of lower trace indicates application of Ca^{2+}-free solution containing 4 mM-EGTA. 0·3 and 10 indicate 0·3 μM- and 10 μM-Ca^{2+} buffered by 2 mM-EGTA. *B*, effects of 1 μM- and 10 μM-GTPγS on the contraction evoked by 0·3 μM-Ca^{2+} with the same procedure as in *A*. *C*, effects of various concentrations of GTP (●) and GTPγS (○) on the contraction evoked by 0·3 μM-Ca^{2+}. $n = 4$–6. * = $P < 0·05$ and ** = $P < 0·01$. *A* and *B* were obtained from different tissues.

dependent manner. The maximum amplitude of contraction was recorded in 10 μM-Ca^{2+} and this amplitude was larger than that recorded in 128 mM-K^+ in the intact muscle tissue (Itoh *et al.* 1983). To prevent the interference by Ca^{2+} released from the SR, 1 μM-ionomycin was present throughout the experiments. Figure 1 shows the effects of GTP and GTPγS on the contraction evoked by 0·3 μM-Ca^{2+}. After the

Ca^{2+}-induced contraction reached a steady level, 100 μM-GTP slightly enhanced the amplitude of contraction (*A*), while 1 μM-GTPγS enhanced the contraction (*B*). Figure 1*C* shows the effects of various concentrations of both agents on the Ca^{2+}-induced contraction (0·3 μM). The amplitude of contraction evoked by 10 μM-Ca^{2+} was normalized as a relative tension of 1·0 (the contractions evoked by 10 μM-Ca^{2+}

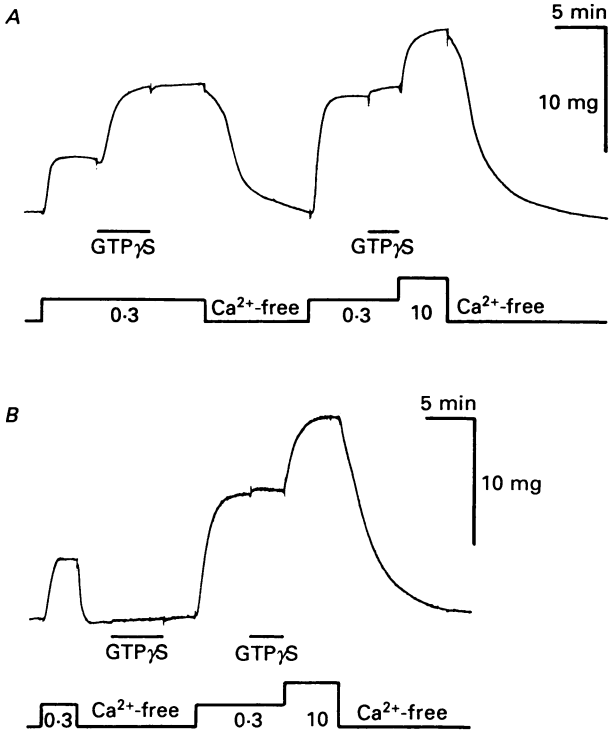

Fig. 2. *A*, irreversible actions of 10 μM-GTPγS on the contraction evoked by 0·3 μM-Ca^{2+} in skinned muscle tissues. Ionomycin (1 μM) was present throughout the experiments. Symbols are the same as in Fig. 1. *B*, effects of 10 μM-GTPγS applied in Ca^{2+}-free solution (2 mM-EGTA) and in 0·3 μM-Ca^{2+}. *A* and *B* were recorded from different preparations.

are shown in Fig. 1*A* and *B*). GTPγS enhanced the amplitude, in a concentration-dependent manner, above 1 μM (in 10 μM, the contraction was 1·92 $\pm$ 0·05 times the control, $n = 4$), whereas GTP only enhanced the contraction in concentrations above 100 μM (in 100 μM, 1·22 $\pm$ 0·04 times the control, $n = 4$).

The enhancing action of GTPγS on the Ca^{2+}-induced contraction was investigated in detail (in the presence of 1 μM-ionomycin). When 10 μM-GTPγS was applied to the contraction evoked by 0·3 μM-Ca^{2+}, the amplitude was enhanced, and on rinsing with relaxing solution containing 4 mM-EGTA, the tissue was relaxed to the resting level. When 0·3 μM-Ca^{2+} was again applied to the tissue, the contraction was markedly enhanced to the same level as observed on application of 10 μM-GTPγS after the 0·3 μM-Ca^{2+}-induced contraction had reached a steady level. Additionally applied 10 μM-GTPγS did not further enhance the amplitude (Fig. 2*A*).

When 10 μM-GTPγS was applied in Ca^{2+}-free solution containing 4 mM-EGTA, no contraction developed, but subsequently applied 0·3 μM-Ca^{2+} enhanced the amplitude as observed in the presence of 0·3 μM-Ca^{2+}. The second application of 10 μM-GTPγS did not further enhance the amplitude of contraction (Fig. 2*B*). This means that the enhancing actions of GTPγS on the force require the presence of Ca^{2+} and the actions of GTPγS are irreversible.

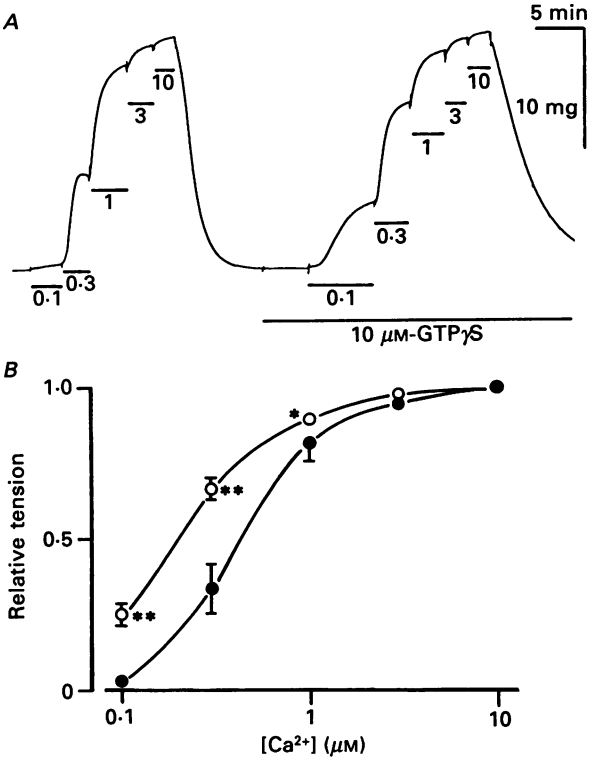

Fig. 3.*A*, effects of 10 μM-GTPγS on the contractions evoked by Ca^{2+}. Various concentrations of Ca^{2+} (from 0·1 to 10 μM) were cumulatively applied after the contraction to each concentration had reached a steady level. The experiments were repeated after application of 10 μM-GTPγS. *B*, the pCa–tension relationship observed before (●) and after (○) application of 10 μM-GTPγS. The amplitude of contraction evoked by 10 μM-Ca^{2+} before application of GTPγS was normalized as 1·0. $n = 3$ preparations. In *A* and *B*, 1 μM-ionomycin was present throughout the experiments. * = $P < 0·05$ and ** = $P < 0·01$.

The pCa–tension relationships were observed in the presence and absence of 10 μM-GTPγS. Stepwise increases in concentration of Ca^{2+} from 0·1 μM to 10 μM were cumulatively applied. As shown in Fig. 3*A*, when 10 μM-GTPγS was applied before and during application of various concentrations of Ca^{2+}, this agent consistently enhanced the amplitude of contraction evoked by Ca^{2+} in concentrations below 1 μM. Figure 3*B* shows the pCa–tension relationship in the presence and absence of GTPγS. The amplitude of contraction evoked by 10 μM-Ca^{2+} in the absence of GTPγS was normalized as 1·0. GTPγS increased the sensitivity of

contractile proteins and enhanced the amplitude with no effect on the maximum response evoked by 10 μM-Ca^{2+}. As a consequence the pCa–tension relationship was shifted to the left.

To clarify whether or not the enhancing action of GTPγS on the contraction required the presence of Ca^{2+}, the effects of GTP and GTPγS were observed on the

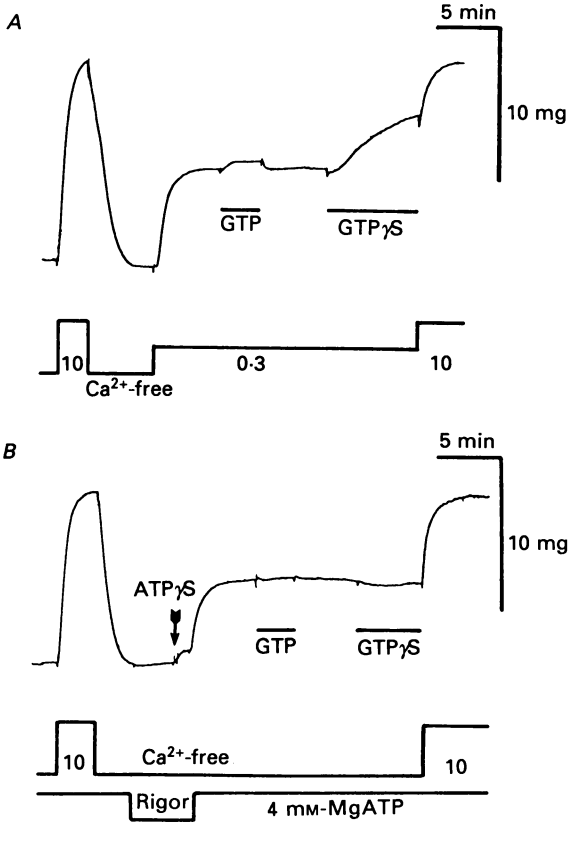

Fig. 4. Effects of GTP (100 μM) and GTPγS (10 μM) on the Ca^{2+}-dependent (*A*) and Ca^{2+}-independent (*B*) contractions evoked in skinned muscle tissues (1 μM-ionomycin was present). As control, the contraction was evoked by 10 μM-Ca^{2+} and then the tissues were rinsed with Ca^{2+}-free solution containing 2 mM-EGTA. Subsequently 0·3 μM-Ca^{2+} was applied (*A*) or Ca^{2+}-free solution rigor solution containing 2 mM-ATPγS was applied (indicated by an arrow) for 30 s then the tissue was perfused with Ca^{2+}-free solution containing 4 mM-Mg-ATP (*B*). After the Ca^{2+}-induced or ATP-induced contraction reached a steady level, 100 μM-GTP and 10 μM-GTPγS were successively applied. 10 μM-Ca^{2+} was then applied in both tissues for comparison with the original control.

Ca^{2+}-independent contraction in skinned muscle tissues. Figure 4*A* shows a control experiment. The contraction evoked by 0·3 μM-Ca^{2+} was slightly enhanced by 10 μM-GTP and markedly enhanced by 10 μM-GTPγS. As shown in Fig. 4*B*, following application of rigor solution (ATP and Ca^{2+}-free solution) for 4·5 min, 2 mM-ATPγS was applied for 30 s. ATPγS produced a contraction of small amplitude, and subsequently applied 4 mM-MgATP produced contraction with the same amplitude

as that observed on application of 0·3 μM-Ca^{2+}. GTP and GTPγS (10 μM) had no effect on the Ca^{2+}-independent contraction. This also supports the view that enhancing actions of GTP and GTPγS on the mechanical response involves the action of Ca^{2+}.

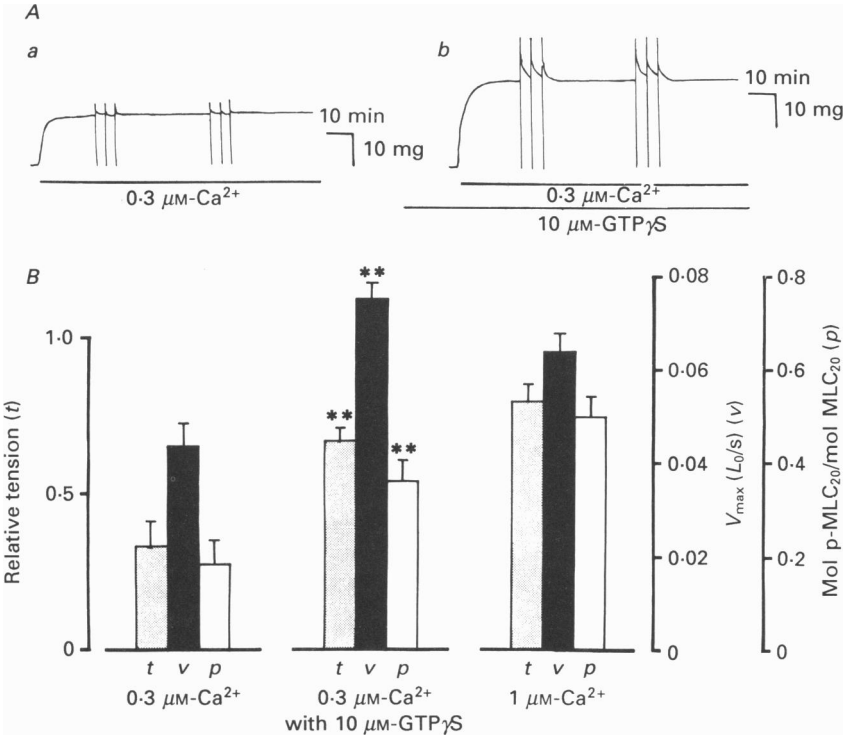

Fig. 5. Effects of 10 μM-GTPγS on the force, V_{max} and phosphorylation of MLC_{20} during treatment with 0·3 μM-Ca^{2+}. Ionomycin (1 μM) was present throughout the experiment. *A*, measurements of shortening velocity of the tissue using the slack test before (*a*) and after application of 10 μM-GTPγS (*b*). Ca^{2+} (0·3 μM) was added 15 min after application of 10 μM-GTPγS. Slack tests were performed 20 and 60 min after application of 0·3 μM-Ca^{2+}. *B*, effects of 10 μM-GTPγS on the force (*t*), shortening velocity (V_{max}; *v*) and phosphorylation of MLC_{20} (*p*) before and after application of GTPγS in the presence of 0·3 μM-Ca^{2+}. These are compared with values observed in the presence of 1 μM-Ca^{2+} in the absence of GTPγS. The amplitude of contraction evoked by 10 μM-Ca^{2+} in the absence of GTPγS was normalized as 1·0. Experimental protocols for measurement of V_{max} and phosphorylation of MLC_{20} are described in the Methods and Results. $n = 4$. * = $P < 0·05$ and ** = $P < 0·01$.

Effects of GTPγS on the contraction, shortening velocity and phosphorylation of MLC_{20}

The effects of 10 μM-GTPγS on the force, V_{max} and phosphorylation of MLC_{20} were observed in the presence of 0·3 μM-Ca^{2+}. In the control, the contraction developed with 0·3 μM-Ca^{2+} was 0·33 ± 0·09 times the 10 μM-Ca^{2+}-induced contraction (control; $n = 4$), and after application of 10 μM-GTPγS the phosphorylation was increased to

0·67 ± 0·04 times the control ($n = 4$). In relaxed skinned muscle tissues, the phosphorylation ratio of MLC_{20} was calculated to be 0·05 ± 0·02 mol p-MLC_{20}/mol MLC_{20}. The amount of phosphorylation was increased, in a Ca^{2+} concentration-dependent manner (0·3 μM-Ca^{2+}, 0·19 ± 0·05; 1·0 μM-Ca^{2+}, 0·51 ± 0·09; 10 μM-Ca^{2+}, 0·62 ± 0·08 mol p-MLC_{20}/mol MLC_{20} measured 20 min after application of Ca^{2+}, $n = 4$–6). In the relaxed condition, phosphorylation of MLC_{20} in the presence of 10 μM-GTPγS was calculated to be 0·06 ± 0·03 mol p-MLC_{20}/mol MLC_{20}, and this value was statistically not significantly different from that observed in the absence of GTPγS. In the presence of 0·3 μM-Ca^{2+}, GTPγS increased the phosphorylation of MLC_{20} from 0·19 ± 0·05 to 0·42 ± 0·08 mol p-MLC_{20}/mol MLC_{20} ($n = 4$).

Figure 5*A* shows traces of measurements of V_{max} during Ca^{2+}-induced contractions in the presence and absence of 10 μM-GTPγS. The V_{max} was measured three times at intervals of 4 min after the Ca^{2+}-induced contraction had reached a steady level. Measurements were taken 20 and 60 min after addition of Ca^{2+} and, since values at 20 and 60 min were the same, the mean value of six individual experiments was obtained. The V_{max} measured in the presence of 0·3 μM-Ca^{2+} (20 min after application of Ca^{2+}) was 0·04 ± 0·009 L_0/s ($n = 4$), and 15 min after application of 10 μM-GTPγS it was increased to 0·072 ± 0·002 L_0/s ($n = 4$). Figure 5*B* summarizes the effects of 10 μM-GTPγS on the force, V_{max} and phosphorylation of MLC_{20} following application of 0·3 μM-Ca^{2+}, and these parameters are compared with those observed on application of 0·3 and 1 μM-Ca^{2+} in the absence of GTPγS.

DISCUSSION

In skinned muscle tissues, 100 nM-A23187 and 1 μM-ionomycin depleted the Ca^{2+} stored in the SR (Itoh, Kanmura & Kuriyama, 1985; Itoh, Kubota & Kuriyama, 1988) and therefore, to observe the effects of GTP and GTPγS on the contractile proteins of the mesenteric artery as estimated from the Ca^{2+}-induced contraction, interference by released Ca^{2+} from the SR was minimized by ionomycin, and Ca^{2+} concentrations prepared for evoking the contraction were buffered with relatively high concentrations of EGTA (4 mM-EGTA; Itoh, Kanmura & Kuriyama, 1985).

Under the above conditions, GTP and GTPγS had no effect on the resting tension but enhanced the contraction evoked by 0·3 μM-Ca^{2+} (GTP, above 100 μM; and GTPγS, 1 μM). Neither agent had an effect on the Ca^{2+}-independent contraction provoked by pre-treatment with ATPγS. Enhancement of the Ca^{2+}-induced contraction was accompanied by an increase in the phosphorylation of MLC_{20} and the shortening velocity (V_{max}; increases in the cyclic rate of cross-bridge formation between actin and myosin; Bárány, 1967; Edman, 1979; Dillon, Aksoy, Driska & Murphy, 1981; Arner & Hellstrand, 1985). These actions of GTPγS on the above parameters observed in 0·3 μM-Ca^{2+} resembled those observed in increased concentrations of Ca^{2+} (1 μM). GTPγS did not modify the maximum amplitude of Ca^{2+}-induced contraction but shifted the pCa–tension relationship to the left and lowered the minimum concentration of Ca^{2+} required to generate the contraction. These results indicate that GTP and GTPγS enhance the contraction induced by Ca^{2+} presumably due to increase in the Ca^{2+} sensitivity of contractile proteins.

Itoh, Kubota & Kuriyama (1986*b*, 1988) observed the effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent activator of protein kinase C, on

smooth muscle tissues of the porcine coronary artery and concluded that this agent increases the Ca^{2+} sensitivity of the contractile protein. Namely, TPA gradually developed the resting tension and enhanced the active tension in high K^+ in intact muscle tissues (below 80 mM) with no change in the Ca^{2+} concentration in the cytosol and also by Ca^{2+} (below 0·5 μM) in skinned muscle tissues. In 128 mM-K^+ or 10 μM-Ca^{2+}, TPA gradually increased the resting tension but inhibited the amplitude of the phasic tension with no transient enhancement. In skinned muscle tissues of the rabbit mesenteric artery, applications of TPA with phosphatidylserine enhanced the tension, shortening velocity and phosphorylation of MLC_{20} induced by 0·3 μM-Ca^{2+} in the same manner as increases in the Ca^{2+} concentrations. However, in the presence of 0·5 μM-Ca^{2+}, such concomitant enhancements of the above three parameters (tension, phosphorylation and shortening velocity) were not observed due to lesser enhancement of the phosphorylation of MLC_{20} than the other two parameters (Fujiwara, Itoh, Kubota & Kuriyama, 1988). Therefore, Fujiwara *et al.* (1988) concluded that TPA increases the Ca^{2+} sensitivity of the contractile protein through activation of the MLC_{20} phosphorylation-dependent and phosphorylation-independent processes.

Although both GTP and TPA seem to increase the Ca^{2+} sensitivity of the contractile protein in smooth muscle cells, different actions of both agents occurred on the mechanical responses, i.e. in skinned muscle tissues, GTP (100 μM) and GTPγS (10 μM) enhanced the Ca^{2+}-induced contraction (0·3 μM) more than TPA (10 nM), while these concentrations of GTP and GTPγS had no effect but TPA markedly enhanced the resting tone. The former did not modify but the latter inhibited the maximum amplitude of contraction evoked by 10 μM-Ca^{2+} (Itoh *et al.* 1986*b*, 1988; Fujiwara *et al.* 1988). These differences in actions of GTP and TPA may indicate that the enhancing action of GTP (GTPγS) on contractile proteins could not be related to activation of phospholipase C (leading to diacylglycerol formation and activation of protein kinase C). Thus, both GTP and TPA may enhance the contraction with different mechanisms.

In conclusion, GTP enhanced the contraction due to increase in the Ca^{2+} sensitivity of the contractile protein. However, the present experiments did not elucidate the actual site of action of GTP or GTPγS on the contractile mechanisms which lead from Ca^{2+}–calmodulin complex to phosphorylation of MLC_{20} (Kamm & Stull, 1985). Reversible and irreversible actions, and weak and potent actions on the Ca^{2+} release induced by GTP and GTPγs, respectively, may partly be caused by hydrolysis of GTP by GTPase and also by reversible binding of GTP to the target protein.

We thank Dr Kate Creed, Murdoch University, Australia, for critical reading of this article. This research was supported by a grant of the Ministry of Education, Japan.

REFERENCES

ABDEL-LATIF, A. A. (1986). Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacological Reviews* **38**, 227–272.

ARNER, A. & HELLSTRAND, P. (1985). Effects of calcium and substrate on force–velocity relation and energy turnover in skinned smooth muscle of the guinea-pig. *Journal of Physiology* **360**, 347–365.

BÁRÁNY, M. (1967). ATPase activity of myosin correlated with speed of muscle shortening. *Journal of General Physiology* **50**, 197–218.

BERRIDGE, M. J. & IRVINE, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321.

BOTTS, J., CHASHIN, A. & YOUNG, H. L. (1965). Alkali metal binding by ethylenediamine tetra-acetate, adenosine 5′-triphosphate, and pyrophosphate. *Biochemistry* **4**, 1788–1796.

CHUEH, S. H., MULLANEY, J. M., GHOSH, T. K., ZACHARY, A. L. & GILL, D. L. (1987). GTP- and inositol 1,4,5-trisphosphate-activated intracellular calcium movements in neuronal and smooth muscle cell lines. *Journal of Biological Chemistry* **262**, 13857–13864.

DAWSON, A. P. (1985). GTP enhances inositol trisphosphate-stimulated Ca^{2+} release from rat liver microsomes. *FEBS Letters* **185**, 147–150.

DAWSON, A. P., COMERFORD, J. G. & FULTON, D. V. (1986). The effect of GTP on inositol 1,4,5-trisphosphate-stimulated Ca^{2+} efflux from a rat liver microsomal fraction: is a GTP-dependent protein phosphorylation involved? *Biochemical Journal* **234**, 311–315.

DILLON, P. F., AKSOY, M. O., DRISKA, S. P. & MURPHY, R. A. (1981). Myosin phosphorylation and crossbridge cycle in arterial smooth muscle. *Science* **211**, 495–497.

DRISKA, S. P., AKSOY, M. O. & MURPHY, R. A. (1981). Myosin light chain phosphorylation associated with contraction in arterial smooth muscle. *American Journal of Physiology* **240**, C222–233.

EDMAN, K. A. P. (1979). The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. *Journal of Physiology* **291**, 143–159.

FUJIWARA, T., ITOH T., KUBOTA Y. & KURIYAMA, H. (1988). Action of a phorbol ester on factors regulating the contraction in the rabbit mesenteric artery. *Circulation Research* (in the Press).

GILL, D. L., UEDA, T., CHUEH, S. H. & NOEL, M. W. (1986). Ca^{2+} release from endoplasmic reticulum is mediated by a guanine nucleotide regulatory mechanism. *Nature* **320**, 461–464.

HAMACHI, T., HIRATA, M., KIMURA, Y., IKEBE, T., ISHIMATSU, T., YAMAGUCHI, K. & KOGA, T. (1987). Effect of guanosine triphosphate on the release and uptake of Ca^{2+} in saponin-permeabilized macrophages and the skeletal-muscle sarcoplasmic reticulum. *Biochemical Journal* **242**, 253–260.

HARAFUJI, H. & OGAWA, Y. (1980). Re-examination of the apparent binding constant of ethyleneglycol-bis-(β-aminoethyl-ether)-*N,N,N′,N*-tetraacetic acid with calcium around neutral pH. *Journal of Biochemistry* **87**, 1305–1312.

HASHIMOTO, T., HIRATA, M., ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986). Inositol 1,4,5-trisphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *Journal of Physiology* **370**, 605–618.

HENNE, V. & SÖLING, H. D. (1986). Guanosine 5′-triphosphate releases calcium from rat liver and guinea pig parotid gland endoplasmic reticulum independently of inositol. *FEBS Letters* **202**, 267–273.

ITOH, T., KANMURA, Y. & KURIYAMA, H. (1985). A23187 increases calcium permeability of store sites more than of surface membranes in the rabbit mesenteric artery. *Journal of Physiology* **359**, 467–484.

ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986*a*). Inorganic phosphate regulates the contraction–relaxation cycle in skinned muscle of the rabbit mesenteric artery. *Journal of Physiology* **376**, 231–252.

ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1986b). Effects of a phorbol ester on acetylcholine-induced Ca^{2+} mobilization and contraction in the porcine coronary artery. *Journal of Physiology* **397**, 401–419.

ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1988). Effects of a phorbol ester on the acetylcholine-induced Ca^{2+} mobilization and contraction in the porcine coronary artery. *Journal of Physiology* **397**, 401–420.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1983). Differences and similarities in the noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. *Journal of Physiology* **337**, 609–629.

KAMM, K. E. & STULL, J. T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annual Review of Pharmacology* **25**, 593–620.

MULLANEY, J. M., CHUEH, S. H., GHOSH, T. K. & GILL, D. L. (1987). Intracellular calcium uptake activated by GTP. *Journal of Biological Chemistry* **262**, 13865–13872.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693–698.

O'FARRELL, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250**, 4007–4021.

PAUL, R. J., DOERMAN, G., ZEUGNER, C. & RÜEGG, J. C. (1983). The dependence of unloaded shortening velocity on Ca^{2+}, calmodulin, and duration of contraction in 'chemically skinned' smooth muscle. *Circulation Research* **53**, 342–351.

RASMUSSEN, H. & BARRETT, P. Q. (1984). Calcium messenger system. An integrated view. *Physiological Reviews* **64**, 938–984.

SAIDA, K. & VAN BREEMEN, C. (1987). GTP requirement for inositol-1,4,5-trisphosphate-induced Ca^{2+} release from sarcoplasmic reticulum in smooth muscle. *Biochemical and Biophysical Research Communications* **144**, 1313–1316.

SATOH, S., KUBOTA, Y., ITOH, T. & KURIYAMA, H. (1987). Mechanisms of the Ba^{2+}-induced contraction in smooth muscle cells of the rabbit mesenteric artery. *Journal of General Physiology* **89**, 215–237.

SCHWARZENBACH, G., SENN, H. & ANDEREGG, G. (1957). Komplexone. XXIX. Ein grosser Chelateffekt besonderer Art. *Helvetica chimica acta* **40**, 1886–1900.

SOMLYO, A. V., BOND, M., SOMLYO, A. P. & SCARPA, A. (1985). Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. *Proceedings of the National Academy of Sciences of the USA* **82**, 5231–5235.

STREB, H., IRVINE, R. F., BERRIDGE, M. J. & SCHULTZ, I. (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306**, 67–68.

SUEMATSU, E., HIRATA, M., HASHIMOTO, T., & KURIYAMA, H. (1984). Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochemical and Biophysical Research Communications* **120**, 481–485.

UEDA, T., CHUEH, S. H., NOEL, M. W. & GILL, D. L. (1986). Influence of inositol 1,4,5-trisphosphate and guanine nucleotides on intracellular calcium release within the N1E-115 neuronal cell line. *Journal of Biological Chemistry* **261**, 3184–3192.